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CH Huang, ME Reid, SS Xie and OO Blumenfeld

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Human Red Blood Cell Wright Antigens: A Genetic and Evolutionary Perspective on Glycophorin A-Band 3 Interaction

By Cheng-Han Huang, Marion E. Reid, Shen-Si Xie, and Olga O. Blumenfeld

The Wright (W_r^a/W_r^b) blood group polymorphism is defined by an allelic change (Lys658Glu) in the band 3 protein; nevertheless, the W_r^b antigen apparently requires glycophorin A (GPA) for surface presentation. To gain insight into the structural basis for this protein-protein interaction and delineate its relationship with W_r^b antigen expression, we investigated GPA and band 3 sequence polymorphisms occurring in rare humans and nonhuman primates. The lack of GPA or amino acid residues 59 through 71 of GPA results in the absence of W_r^b from human red blood cells (RBCs) exhibiting the M^hM^h , $En(a-)$, or MiV phenotype. However, the SAT homozygous cells carried a Glu₆₅₈ form of band 3 and a hybrid glycophorin with the entire GPA extramembrane domain from residues 1 through 71, yet expressed no W_r^b antigen. This finding suggests that formation of the W_r^b antigenic structure is dependent on protein folding and that the trans-

membrane junction of GPA is important in maintaining the required conformation. Comparative analyses of GPA and band 3 homologues led to the identification in the interacting regions of conserved and dispensable amino acid residues that correlated with the W_r^b positive or negative status on nonhuman primates. In particular, the chimpanzee RBCs cells expressed W_r^b and the Glu₆₅₈ form of band 3, which is identical to humans, but their GPA contained the Gly rather than Arg residue at position 61. Taken together, the results suggest that (1) Arg₆₁ of GPA and the proposed Arg₆₁-Glu₆₅₈ charge pair are not crucial for W_r^b antigen exhibition and (2) the role of GPA for interaction with band 3, including Glu₆₅₈, probably involves a number of amino acid residues located in the α -helical region and transmembrane junction.

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SINCE FIRST DESCRIBED,¹ the Wright (W_r) antigens in human red blood cells (RBCs) have attracted an intensive series of investigations.² There are two blood group antigens, the low-incidence W_r^a and the high-incidence W_r^b , that are considered to be antithetical and are produced as allelic forms of the same structural gene.^{3,4} Despite the fact that alloimmunization may cause transfusion reactions and hemolytic disease of the newborn,⁵ the functional significance of W_r antigens, if any, remains unknown. Furthermore, these antigens appear to be dispensable because no morphologic or functional abnormalities are manifested in human RBCs lacking their expression [$W_r(a-b-)$].^{6,8}

Over the years, interest in the W_r antigens has centered on their identity and on requirements for their surface presentation. Studies have focused on an interaction between glycophorin A (GPA) and band 3 (the anion exchanger), the two RBC transmembrane (TM) proteins in similar abundance.^{9,10} Evidence for the involvement of GPA first came from the finding that GPA-deficient $En(a-)$ RBCs did not display the W_r antigens.⁶ Immunoprecipitation and reconstitution studies also indicated that the W_r^b antigen requires GPA and lipids for reactivity.^{11,12} Proteolysis and chemical modification experiments suggested that amino acids 62 through 70 of GPA, a putative helical region,¹¹ engage the labile struc-

ture of the W_r^b antigen.¹² However, the independent segregation of W_r^a from GPA-borne M/N blood group antigens¹³ and no apparent change in the residues 40 through 96 of GPA among $W_r(a+b-)$, $W_r(a+b+)$ and $W_r(a-b+)$ individuals¹² suggested that the W_r antigens most likely involve an additional membrane component.

It was postulated that band 3 could be the candidate and its interaction with GPA might be responsible for the disposition of W_r antigens in the RBC membranes.¹⁴ This view conciliated with the altered glycosylation of band 3 in $En(a-)$ RBCs,¹⁵ the decreased rotation of band 3 induced by anti-GPA antibodies,¹⁶ and the aggregation of band 3 with GPA in the presence of Triton X-100 detergent.¹² Further support for the hypothesis came from more recent studies showing the coprecipitation of band 3 and GPA by anti- W_r^b antibodies^{17,18} and the facilitation by GPA of band 3 expression in the *Xenopus* oocyte system.¹⁹ Most recently, the W_r^a/W_r^b polymorphism was shown to result from an allelic change (A1972G) in the band 3 gene causing the Lys658Glu substitution.²⁰ This finding, together with the knowledge about natural variations of glycophorin,^{21,22} suggests a model in which Glu₆₅₈ of band 3 interacts with Arg₆₁ of GPA to form the W_r^b antigen.²⁰ We examine here the model from a genetic evolutionary perspective and present data showing how structural changes in GPA and band 3 might reshape the protein-protein interaction and thus affect W_r^b antigen expression.

MATERIALS AND METHODS

Blood samples and serologic testing. Blood samples used as controls were obtained from normal human blood donors. Blood samples exhibiting variant phenotypes of the MNS blood group system were gifts from the following sources: M^hM^h , $En(a-)$, and SAT bloods were from the Osaka Red Cross Blood Center (Osaka, Japan); MiV blood from the American Red Cross (Los Angeles, CA); Dantu and S-s-U- bloods were from the Community Blood Center (Dayton, OH); and St^h blood was from the Miyagi Red Cross Blood Center (Sendai, Japan). Blood samples of nonhuman primates were provided by the Wildlife Conservation Society (Bronx, NY), the Yerkes Primate Center (Atlanta, GA), or the Laboratory for Experimental Medicine or Surgery in Primates (Tuxedo, NY). The

From The Lindsley F. Kimball Research Institute, New York Blood Center, New York, NY; and the Department of Biochemistry, Albert Einstein College of Medicine, Bronx, NY.

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Address reprint requests to Cheng-Han Huang, MD, PhD, Lindsley F. Kimball Research Institute, New York Blood Center, 310 E 67th St, New York, NY 10021.

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Wr^b antigen status on RBCs of animals was tested with human antiserum (MF) using standard hemagglutination techniques.

Isolation of DNA and RNA. Genomic DNAs were prepared from peripheral blood leukocytes as described.²⁵ Total RNA was isolated from hemolysates using a sequential cell lysis method²⁴ and then extracted with Trizol reagent (BRL, Gaithersburg, MD).

Synthesis and amplification of cDNA. Synthesis and amplification of cDNAs from erythroid total RNA was performed by reverse transcription-coupled polymerase chain reaction (RT-PCR), as previously described.²⁵ To obtain band 3 cDNA products, two primers that define, respectively, exons 16 and 17 of the human gene were used: AE1, 5'-AAACTCTCGGTGCTGATGGCTTC-3' (nt 1891-1914, sense), and AE2, 5'-GAGCCCTTGACCATCTTGCGCTCA-3' (nt 2076-2099, antisense).²⁶⁻²⁸

To obtain GPA cDNAs from higher primates, the following human primers were used:^{25,29} GP1, 5'-GTATGGAAAAATAATCTT-TGTATTAC-3' (nt 3-28, exon 1 for signal peptide, sense); GP2, 5'-AGCATATCAGCAT(C/T)AAGTACCAC-3' (nt 46-69, exon 2, sense); GP3, 5'-ATCACTTGTCTCTGGATTTTCTATTTC (nt 421-447, exon 6-7, antisense); and GP4, 5'-TCCACATTTGGTTTG-GTGAACAGATTC-3' (nt 454-480, exon 7, antisense). GP3 and GP4 could only prime the synthesis of GPA cDNAs, because their sequences are located in the last two exons encoding the cytoplasmic domain and are not present in the GPB gene.^{25,29} After first-strand synthesis, the GPA cDNA product was amplified 30 cycles in 50 μ L volume on a thermocycler (Ericomp, San Diego, CA). The first 29 cycles were each run at 94°C for 1 minute, at 55°C for 45 seconds, and at 72°C for 1 minute. For the last cycle, annealing at 55°C and chain extension at 72°C was for 2 and 7 minutes, respectively.

Amplification of genomic DNA sequences. Exon 16 of the band 3 gene spanning the A1972G (Lys658Glu) polymorphism was amplified from total genomic DNA in the presence of two primers, AE1 (see above) and AE3 (5'-TCTCACGTGGTGATCTGAGACTCC-3').²⁸

DNA sequence determination. The PCR-amplified cDNA and genomic DNA products were purified by native 5% polyacrylamide gel electrophoresis and their nucleotide sequences were directly determined on an automated DNA sequencer (Applied Biosystem, Foster City, CA).

RESULTS

Band 3 and GPA expression in Wr(a-b-) and Wr(a-b+) individuals with MNSs-related variants. To further delineate the structure-phenotype relationship for the Wr^b antigen, the band 3 polymorphism and glycophorin expression were revisited in 7 individuals with MNSs-related variant phenotypes. These phenotypes included M^kM^k, En(a-), S-s-U-, St^a, MiV, Dantu, and SAT. Of the 7 individuals examined, all but the Dantu-positive proband were homozygotes. RBCs from these individuals were either deficient in glycophorins^{23,30-32} or associated with expression of hybrid glycophorins resulting from unequal crossovers.³³⁻³⁷ As determined by sequencing of PCR-amplified cDNA or genomic DNA products, all individuals carried the Glu₆₅₈ but not the Lys₆₅₈ form of band 3 (data not shown), indicating that they were all homozygotes for the Wr^b allele. In M^kM^k, En(a-), and S-s-U- cells, the Wr^b status apparently paralleled the absence or presence of GPA and correlated with the genetic status of the GPA gene. For RBCs bearing glycophorin hybrids except GPSat, the pattern of Wr^b antigen expression complied with the absence or presence of the GPA portion ex-

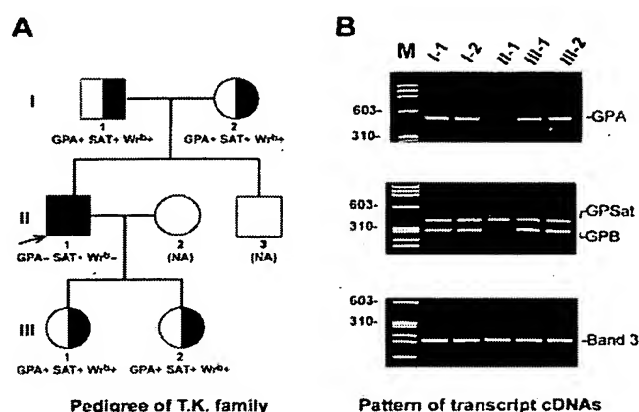


Fig 1. RT-PCR analysis of glycophorin and band 3 expression in the SAT(T.K.) family. (A) Pedigree of the family in which the status of GPA and SAT and Wr^b antigens is indicated. Note that proband II-1 (indicated by arrow) was homozygous for the GPSat gene. NA, individuals not available for this study. (B) Pattern of transcript cDNAs for glycophorins and band 3 from the family members. The amplified cDNA products were separated on native 5% to 7% polyacrylamide gel electrophoresis and stained with ethidium bromide. The size of ϕ X174 DNA Hae III markers (M) and the identity of cDNA species are indicated at the left and right margins, respectively. Note that neither GPA nor GPB product is seen in the SAT homozygote who lacked the Wr^b antigen on the RBC surface.

pressed (see below). These data showed that, without GPA, the Glu₆₅₈ form of band 3 alone does not display the Wr^b determinant.

Expression and inheritance of the Wr^b antigen in SAT (T.K.) family. SAT is a private RBC antigen associated with the expression of two glycophorin isoforms in different families.³⁸ In the T.K. family, expression of the SAT antigen was accompanied by the inheritance of a GPA-B hybrid gene, GPSat, that arose via a similar mechanism as GPMiV but differed from the latter in the site of crossover point.³⁷ RT-PCR analysis of this three-generation family showed a genetic association but a phenotypic dissociation of band 3 with the Wr^b antigen. The erythroid cells from the five SAT-positive members all contained the band 3 and GPSat transcripts; nevertheless, in contrast to the heterozygotes, the homozygote (donor II-1) lacked both GPA and GPB transcripts (Fig 1). Although sequencing of the band 3 cDNAs showed that all individuals should be the Glu₆₅₈ homozygotes, the Wr^b antigen was segregated from GPSat in donor II-1 but was cotransmitted with GPA in members of the first and third generations (Fig 1). This inheritance pattern reinforced the hypothesis that the Wr^b antigen requires a specific interaction between the band 3 and GPA proteins.^{14,17,20}

Conformational dependence of the Wr^b antigen and role of GPA TM junction. It has been suggested that the region of GPA, from residues 62 through 70, involves the Wr^b antigen and forms part of an α -helix immediately adjacent to the TM segment.^{11,12} Definition of the sequence as part of the Wr^b epitope is supported by the absence of the Wr^b anti-

GP	Wr ^b Status	Hybrid Arrangement	GPA moiety of the Wr ^b epitope	
GPA	+	-	51 VYPPEEETGERVQLAHHFSEP	EITLI 76
GPB	-	-	19 SYISSQTNGETGQLVHRFTVPAPVVILLI	47
GPSt ^a	+	B1-26/A59-131	19 SYISSQTNGERVQLAHHFSEP	EITLI 44
GPMiV	-	A1-58/B27-72	51 VYPPEEETGETOQLVHRFTVPAPVVILLI	79
GPDantu	-	B1-39/A71-131	19 SYISSQTNGETGQLVHRFTVP	EITLI 44
GPSat	-	A1-70/B40-72	51 VYPPEEETGERVQLAHHFSEPAPVVILLI	79
			TM Junction	

Fig 2. Comparison of the amino acid sequences between the parent and hybrid glycoproteins in the region relevant to Wr^b antigen exhibition. GPA and GPB, the parent molecules, are shown above GPSt^a, MiV, Dantu, and Sat, the hybrid molecules. The GPA sequence that may engage the labile structure of the Wr^b antigen is overlined. GPB and the GPB-derived sequences are shaded. The arrangement of hybrids with respect to the GPA and GPB polypeptides and their status associated with Wr^b are shown. Note that GPSt^a versus GPMiV and GPDantu versus GPSat are two pairs of reciprocal protein products whose crossing-over points in the genes reside in introns 3 and 4, respectively. The TM junction that interfaces the extracellular and membrane-spanning domains of glycoproteins is indicated.

gen in MiV RBCs that express a GPA-B hybrid lacking GPA residues 59 through 71^{35,36} (Fig 2).

However, the occurrence of linear sequences involving the interaction may not always lead to Wr^b antigen expression. As shown, the SAT homozygous RBCs expressed the Glu₆₅₈ form of band 3 and the hybrid glycoprotein, GPSat (Fig 1). Comparison of the amino acid sequences (Fig 2) shows that the hybrid structure of GPSat is reciprocal to that of GPDantu^{39,40} and distal to that of GPMiV^{35,36} or GPSt^a.⁴¹ Thus, GPSat retains the entire extramembrane domain of GPA from residues 1 through 71, including the moiety for the Wr^b epitope, and differs from GPA in the extramembraneous junction and TM segment (Fig 2). The lost Wr^b expression in the SAT homozygote strongly suggests that formation of the epitope depends on proper contact of the two proteins and that the TM junction of GPA plays an important role in maintaining the required conformation. This notion is consistent with our recent studies showing the conformational dependence of the S, s and U antigens and the importance of the GPB TM junction for their presentation.⁴² In the case of GPSat, it is likely that the insertion of three GPB residues (Ala-Pro-Val) at the TM junction could readjust the orientation of the preceding α -helix and thus alter the native antigen structure necessary for anti-Wr^b binding. This is different from GPSt^a or GPMiV, in which the retention or loss of the Wr^b epitope sequence (Fig 2) has caused a coprecipitation and a null reaction with the anti-Wr^b antibody, respectively.^{11,43}

Sequence polymorphisms of GPA and band 3 protein homologues in nonhuman primates. Among nonhuman primates, only chimpanzee RBCs express the Wr^b antigen at a level comparable to humans, whereas RBCs from orangutans, gibbons, and rhesus monkeys essentially lack the serologic reaction.^{44,45} Because the definition of such positive or negative status could show the underlying structural diversity, we determined the Wr^b antigen status on animal RBCs

and sequenced their cDNAs encoding the homologues of GPA and band 3 proteins. Comparison of the deduced primary sequences encompassing the Wr^b domain (Fig 3) showed that (1) the GPA homologues are more divergent

GPA						
		Wr ^b	51	61	Wr ^b	TM 82
Hu	+	VYPPEEETGER	VQLAHHFSEPEITLI	IFGVMA	100	
Ch-1	+	-----N--G	---V-R-----	-----	88	
Ch-2	+	-----V-N--G	---V-R-----	-----	84	
Or-1	-	THS-----N--	G---V-R-----V--V--	-----	72	
Or-2	-	IHS-----N--	G---V-R-----V--V--	-----	72	
Gib	-	-----D--WGQG	---V-R-----V--V--	-----	75	
Rh	-	H-----DNR--	---V-E-----LV-A--	-----	72	
Mo	NT	AIHVSTYHTAPTE--	---D-PVLVMI---	L-----	34	

Band 3						
		Wr ^b	640	658	TM8 671	
Hu	+	VSNESARGWV	IHPLGLRSEFPI	WMMFASALPA	100	
Ch-1	+	-----	-----	-----	100	
Ch-2	+	-----	-----	-----	100	
Or-1	-	-----S-----	-----H-----	-----	94	
Or-2	-	-----S-----	-----H-----	-----	94	
Gib	-	-----S-----	-----H-----	-----	94	
Rh	-	-----I-----	-----H-----	-----	91	
Mo	NT	-----	-----WRL--T-----	-----V-----	84	

Fig 3. Deduced amino acid sequences of GPA and band 3 protein homologues from nonhuman primates. (Top) GPA sequences. For clarity, only those amino acid residues corresponding to positions 51 through 82 of human GPA are shown. Amino acid differences are spelled out and identical residues are denoted by dashes. (Bottom) Band 3 sequences. Sequences corresponding to residues 640 through 671 of human band 3 are aligned. The N-glycosylation consensus sequence NSS is shaded. Designation of species: Hu, human; Ch, chimpanzee; Or, orangutan; Gib, gibbon; Rh, rhesus; and Mo, mouse. Ch-1, Ch-2, and so on denote unrelated individuals. The deduced sequences of the two proteins were obtained from the same animals, except those for mouse glycoprotein⁴⁶ and mouse band 3.⁴⁷ Arg₆₁ of GPA and Glu₆₅₈ of band 3 are indicated by vertical arrows. The sequences tentatively assigned as the interacting domain for Wr^b are overlined and so are the portions of the TM segment of the two proteins. The Wr^b antigen status on RBCs of each animal is shown at left: +, positive; -, negative; and NT, not tested. The percentage of identity of the animal sequence relative to the human sequence (100%) is indicated at the right margin.

than the band 3 homologues in different species, but the former have a number of conserved amino acids in positions 62 through 71, including Gln₆₃Leu₆₄, His₆₆, and Phe₆₈Ser₆₉. Glu₇₀; and (2) both Arg₆₁ of GPA and Glu₆₅₈ of band 3, the two residues thought to be critical in forming the Wr^b antigen,²⁰ are variable from rhesus to chimpanzee.

As shown for GPA sequences (Fig 3, top), Arg₆₁ was present in the orangutan and rhesus but was replaced by Gly and Trp in the chimpanzee and gibbon. Scattered changes in positions 51 through 82 included unique insertion or random mutations, but positions 65 and 67 were occupied by reiterated substitutions in different species. Accordingly, Val₁₆₅Ala and Arg₆₇His distinguish chimpanzee from human. In orangutan or gibbon, one more change, Val → Gly₆₂, made the GPA sequence in positions 62 through 67 be identical with the human GPB sequence in positions 30 through 35 (Gly-Gln-Leu-Val-His-Arg; Figs 2 and 3). In the rhesus, its GPA differed from human GPA by three residues in positions 62 through 71 (Val₆₅Ala, Glu₆₇His, and Leu₇₁Pro; Fig 3). In mice, a low sequence identity (34%) is mainly confined to the TM segment and there is no significant homology in the Wr^b domain.⁴⁶

Regarding the band 3 sequence (Fig 3, bottom), the region encompassing the extracellular loop and the adjacent membrane-spanning segment (TM 8th pass) is well conserved and, even in mice,⁴⁷ the sequence identity is as high as 84%. In that region, the chimpanzee had an identical sequence with humans. The orangutan/gibbon and rhesus/baboon pairs each shared the same sequence, with two or three substitutions located between the N-glycosylation site and TM 8 (data for baboon not shown). Of all animals examined, no Lys₆₅₈ form of band 3 was detected, suggesting that the Wr^b antigen may also be rare in nonhuman primates. Nevertheless, Glu₆₅₈ occurred in chimpanzees, but it had been replaced by a histidine residue in other species.

Correlation of sequence variation with Wr^b antigen expression in nonhuman primates. It is evident that the proposed Arg₆₁-Glu₆₅₈ charge pair²⁰ cannot be formed in the animals studied due to the substitution of either one or both residues (Fig 3). This finding suggests that Arg₆₁ of GPA is dispensable or replaceable with respect to the Wr^b reactivity, at least in the case of chimpanzees. Comparable Wr^b expression in human and chimpanzee RBCs (Fig 3) also implied that the two other changes in the helical region, Ala → Val₆₅ and His → Arg₆₇, may not affect the overall structure of the antigen. In the orangutan and gibbon, the absent Wr^b expression was apparently correlated with changes of Glu → His₆₅₈ on band 3 and Val → Gly₆₂ on GPA, because the former removed a negative charge and the latter rendered the sequence more like GPB, which is known not to display the Wr^b reactivity (Fig 2). Similarly, the lack of Wr^b expression on rhesus RBCs could also be attributed to changes on both band 3 (Glu → His₆₅₈) and GPA. Regarding the rhesus GPA moiety, the Arg₆₇Glu change introduced a negative charge, and the substitution by Leu of conserved Pro₇₁, a helix breaker, might perturb the local conformation and thus alter the interaction. It is notable that, Ser₆₉Glu₇₀ in the TM junction of GPA may also be important for the GPA-band

3 interaction, because they differ from human GPB (Fig 2) but are conserved from the rhesus to humans (Fig 3, top).

DISCUSSION

In contrast to the Wr^a antigen that may be formed by band 3 alone, there is considerable evidence indicating the dependence of Wr^b antigenicity on a specific GPA-band 3 interaction.¹²⁻¹⁹ Nevertheless, the nature of this protein-protein interaction as well as the role of individual amino acid residues in Wr^b antigen formation remains largely speculative. The difficulty in defining this interaction stems partly from the fact that single amino acid changes in the regions encompassing GPA residues 62 through 70 and band 3 residues 651 through 660 are seldom encountered in the human populations, despite the recent finding of a rare Lys₆₅₈ polymorphism on the band 3 protein.²⁰ This report describes two novel observations concerning the expression of the Wr^b antigen. The first observation is that the alteration of TM junction in GPSat silences the human antigen. The second observation points to a correlation of the Wr^b status with sequence variations on GPA and band 3 homologues in non-human primates. These data provide new insights, in the context of primary structures, into the molecular basis for the GPA-band 3 interaction and its relationship with Wr^b exhibition.

Of the Wr^b null phenotypes found in human RBCs, the one associated with SAT homozygous cells is particularly intriguing in that GPSat retains an apparently intact sequence essential for the antigen presentation. The antigen disruption in GPSat by a small insertion distal to the putative Wr^b domain raises the possibility that the extramembranous junction or adjacent TM residues participate in or influence the GPA-band 3 interaction. Although both contain the Wr^b moiety, GPSat makes a contrast with GPS^a, because the latter expressed the antigen weakly¹¹ and carried a GPB sequence⁴¹ proximal to the residues 62 through 70. This comparison indicates that the Wr^b antigen is sensitive to local structural changes and that the closer the alteration is to the TM segment, the more profound the conformational perturbation.

The definition of Wr^b positivity or negativity in nonhuman primates by inspection of sequence divergence and conservation broadens our view on the human counterpart from an evolutionary perspective. Our data suggest that Glu₆₅₈ of band 3 and VQL₆₂₋₆₄, His₆₆, and FSEP₆₉₋₇₁ of GPA are important for the GPA-band 3 interaction and Wr^b antigen exhibition, whereas Arg₆₁, Ala₆₅, and His₆₇ are not so crucial. Although scattered amino acid variations proximal to position 58 of GPA occur in animals (Fig 3), they are unlikely to be crucial either, because GPSt^a carries a completely different sequence proximal to that position (Fig 2), yet still displays the Wr^b reactivity.¹¹ Apparently, the role of the GPA moiety for epitope formation mainly involves amino acids that are located in the helical region and TM junction. In addition, the associated Wr^b expression in the absence of Arg₆₁, but presence of Arg₆₇, in chimpanzee GPA suggests that the overall surface charge of the α -helix, rather than the specific location of Arg residues, would be more important, if the ionic interaction with Glu₆₅₈ of band 3 occurs at all.

Because parallel packing of the TM α -helices of the two proteins would bring the preceding extramembranous portions in close proximity, the helical region of GPA may stabilize the interaction and therefore the W_r^b antigen by forming additional contacts with the residues nearby Glu₆₅₈ of the band 3 protein.

In summary, the present studies have provided evidence for the conformational dependence of the W_r^b antigen and led to the identification of certain amino acid residues that may be important for its exhibition. Nevertheless, although the W_r^b antigen may be considered a phenotypic indicator of the GPA-band 3 interaction, its lost expression does not necessarily mean the abolition of the interaction. How those local changes cause the phenotypic silencing of W_r^b and whether they reshape the interaction to elicit new antigenicities, such as SAT and S_r^b , are some of the issues that remain to be investigated. Studies combining site-directed mutagenesis with cotransfection of GPA and band 3 cDNAs in an *ex vivo* system should allow one to dissect in depth the structural elements involved in such protein-protein interactions.

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